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Detectors: Electron Spin Resonance

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Introduction

The role of radicals in (bio-)chemical reactions is currently becoming increasingly significant. Free radicals may be generated by any kind of irradiation and contribute essentially to many aging processes in many materials, particularly in the presence of oxygen. They are even able to cause manifold organic damages as in lipid peroxidation or in inflammatory diseases. Organic reperfusion injuries after ischaemia are currently the subject of intensive research activities. On the other hand, stable free radicals are used practically in a number of applications in many fields, e.g. as additives in industrial processes such as polymerization or as analytical tools in research on membrane, emulsion and surface properties of materials or formulations. The utilization of such substances as protective additives, e.g. for process control or as research tools, has stimulated interest in the synthesis of new compounds of this class. The increasing search for radicals is paralleled with a rising demand for methods to detect, identify and quantify them. In the context of separation techniques, this means having a technique at hand to trace them in eluted fractions.

High chemical reactivity combined with low specificity is typical of the majority of radicals. As a consequence, solutions containing such substances are likely to alter their composition within a short time owing to the decreasing content of reactive components and to the accumulation of reaction products. This kind of change can easily be monitored by chromatographic methods. Having separated all the constituents at a given time, it may become necessary to identify original or intermediate radicals to evaluate the particular stage of an ongoing reactive process. However, the detectors routinely used in HPLC cannot indicate directly any radical present in the separated fractions. The most advanced method suitable for this purpose is electron spin resonance spectroscopy (ESR), but most ESR spectrometers are currently installed as large and heavy instruments, not at all suitable as detectors for chromatographic methods. It will be shown here that this kind of spectroscopic method can be realized with devices of table-top size that can easily be integrated into any chromatographic separation line.

Electron spin resonance (or electron paramagnetic resonance, EPR) spectroscopy is the only direct method to measure radicals since it is based on the existence of unpaired electrons. Likewise, paramagnetic metal complexes are also sensitive to this spectroscopic method. A substantial advantage for the study of radical reactions would be the rapid analysis of a fraction directly upon separation to avoid changes caused by putative consecutive reactions. This can only be achievable by direct coupling of the separator (HPLC) with the specific detector (ESR) in the shortest possible way without any unnecessary dead volume (long tubing lines or valves). An absolute prerequisite for such an instrumental set-up is a spectrometer of a size that allows installation at the site of the sample separation, and not necessarily vice versa. The other problem is synchronization of sample separation and recording of a spectrum which requires a definite period of time. Both problems have been solved in the on-line coupling of HPLC and ESR spectroscopy described here.

ESR Spectroscopy as Detector

An introduction into the principles of ESR (or EPR) spectroscopy can be found in most textbooks on physical chemistry or in specialized monographs. Since this spectroscopic technique is rather uncommon

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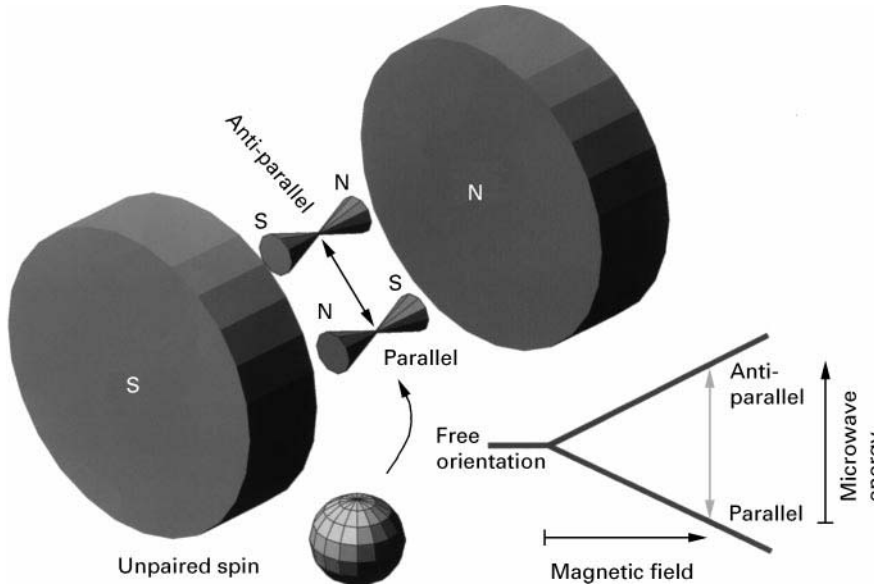

Figure 1 Alignment of an unpaired electron in a magnetic field.

in the field of separation sciences, a brief description of the principle is given here.

Electron spin resonance was detected by the Russian scientist Zavoisky in 1946 while researching the paramagnetic properties of matter. Radicals and metal complexes with an odd number of electrons in d-orbitals show paramagnetism since unpaired electrons generate local electromagnetic fields. They can be detected whenever they interact with an external magnetic field. This interaction can be understood in the way that the external field forces any unpaired electron to align (**Figure 1**).

Once in line with the parallel magnetic field, lines of a given strength the electrons are able to flip into the opposite direction while absorbing energy. The stimulating energy ($\Delta E = h\nu$, h is Planck's constant and ν is the frequency) to cause such a flip over must be of a frequency able to resonate with the unpaired electron tumbling in the magnetic field like a spinning top (Larmor frequency). This frequency again is linearly dependent on the applied magnetic field; in other words, the stronger the field, the higher must be the frequency of the energy to stimulate the flip over of a single electron. ($\Delta E = g\mu_B H$ where μ_B represents the Bohr magneton, H the external magnetic field and g is a dimensionless proportional factor, ~ 2 for a single electron, the so-called Landé factor. The whole term is part of the Hamiltonian operator which is used to describe the state of energy of a given system.)

In essence, the alignment of an unpaired electron in an external magnetic field can have two directions, either with the field (low-energy state) or against the field (high-energy state). This is the same kind of energy splitting (Zeeman splitting) as in nuclear magnetic resonance (NMR) spectroscopy. The transition between these two states needs energy in the range of high-frequency radio waves or microwaves, depending on the strength of the applied external magnetic field. Nowadays, it is quite common to use a microwave frequency between 9 and 10 GHz (within the so-called X-band) which requires a magnetic field in the range of 300–400 mT. To find the exact absorption energy of an unpaired electron, e.g. in a radical at a given strength of the applied magnetic field, two ways are possible in principle: either to scan the microwave at a fixed magnetic field, or vice versa, to scan the magnetic field at a fixed wavelength. Since it is technically much easier to vary the magnetic field rather than to tune a microwave source over a large range, ESR spectra are recorded by scanning the external magnetic field. They are commonly displayed as first derivatives which is different to all other spectroscopic techniques where absorption peaks are shown.

Interactions of unpaired electrons with surrounding paramagnetic nuclei result in additional splitting of energy levels (hyperfine splitting) that become visible as multiples of absorption lines. In some cases, different radical moieties can be identified by their individual hyperfine splitting structure.

Coupling between Radical Separation and Detection

The application of ESR spectroscopy as a detector in a separation technique like HPLC raises a number of problems which have been solved in the following way.

ESR Spectrometer Size

Common ESR spectrometers have a large magnet which makes the instruments rather heavy, up to half a ton in weight. However, newly developed ESR spectrometers have been reduced to a size comparable with commonly used diode array detectors.

Flow Conditions of HPLC Mobile Phases

Owing to the properties of the microwave, the sample size is geometrically restricted to a thickness of 0.3 mm, allowing only a total sample volume of some 50 μL (if the fluid phase contains water, the volume could be larger otherwise). The sample cuvette (a quartz flat cell) is located in a fixed position inside the instrument having open access from the bottom and top allowing tubing to be attached to fill the cuvette upwards and to flow out at the top of the instrument. This vertical flow direction of the mobile phase helps to avoid accidental trapping of gas bubbles that could spoil any spectral measurement. At a flow of 1 mL min^{-1} no turbulence occurs at any site within the sample cuvette ensuring homogeneous filling and flushing without any problem. The use of capillary tubing minimizes the dead volume between the common UV/visual light monitor and the ESR spectrometer and thus the flow delay between these two devices.

Tubing Material Required for Radical Detection

In all cases, non-metal tubing is used. It should be noted that steel capillaries can never be used in the vicinity of the magnetic field of the spectrometer. Moreover, it is advisable to avoid any metal surface at all when working on free radicals. Particularly if oxygen is present, even minute amounts of iron, for example, may be sufficient to catalyse the Fenton reaction.

Synchronization of Separation and Detection

A major problem to be solved is synchronization of the continuous sample flow from the chromatographic equipment and the scan time required for taking an ESR spectrum. This subject has already been tackled by a number of authors. However, problems regarding signal separation and measurement sensitivity remain. A typical ESR scanning time is one minute for a spectrum and this may be in conflict with the elution time of a single fraction from a HPLC column. Since complete chromatograms usually take some 8–15 min, single fractions are likely to take less than a minute to elute so no constant conditions can be expected in the sample cuvette while a spectrum is running. Simply stopping the flow for the time of recording a spectrum may result in local diffusion, causing a loss of resolution in the separation step, so this approach is not recommended. Pulsed short-time spectra (FT-spectroscopy) are very sophisticated requiring a much more complicated instrumentation, so the 'cw' (continuous wave) technique still remains the current standard method.

Instrumental Arrangement and Operation

A practical solution that allows both continuous flow from the HPLC column and a resting period of a sample fraction in the ESR spectrometer cuvette is depicted in **Figure 2**. In principle, a shunt is provided to short-cut the passage through the ESR spectrometer. It is opened while an ESR spectrum is in progress and closed at any other time. This allows the cuvette to be filled with a fraction, to leave it for the time of scanning a spectrum, and to flush the sample upon completion. A disadvantage of this procedure is that is does not allow spectra of fractions running closely together to be obtained since the spectrum of the first sample is still being recorded while the second one is starting to elute from the column. In this case, it is necessary to repeat the chromatographic separation step for the second fraction of interest.

In order to control the flow either through or bypassing the ESR spectrometer, a T-connector is inserted into the tubing between the HPLC detector and the ESR spectrometer for branching off to the shunt. Both the outlet tubing from the spectrometer and the shunt are connected to a valve which alternately opens or closes either way. This arrangement has two major advantages: on the one hand, unnecessary and irregularly shaped dead volume that may disturb the sample flow from the HPLC device to the spectrometer is avoided by placing the control valve after both devices, and on the other hand, it is not possible to close off both paths by control error, thus minimizing the risk of accidentally building up destructive pressure in the tubing system. In a simple set-up, the valve can be operated by hand via an electrical switch. The delay time from the appearance of the peak of interest in the monitor system of the HPLC to the moment of filling the ESR cuvette with the corresponding fraction can be measured exactly and is highly reproducible. In more standardized or routine applications, automatic control is possible. This whole arrangement makes the ESR spectrometer truly an additional HPLC monitor that analyses radicals.

Supplementary Equipment

In some cases substances may turn into (short living) radicals after stimulation either by light irradiation or in the course of a reaction triggered by an added reagent. In other cases, reactive and short-lived radicals may be trapped in an adduct with a so-called

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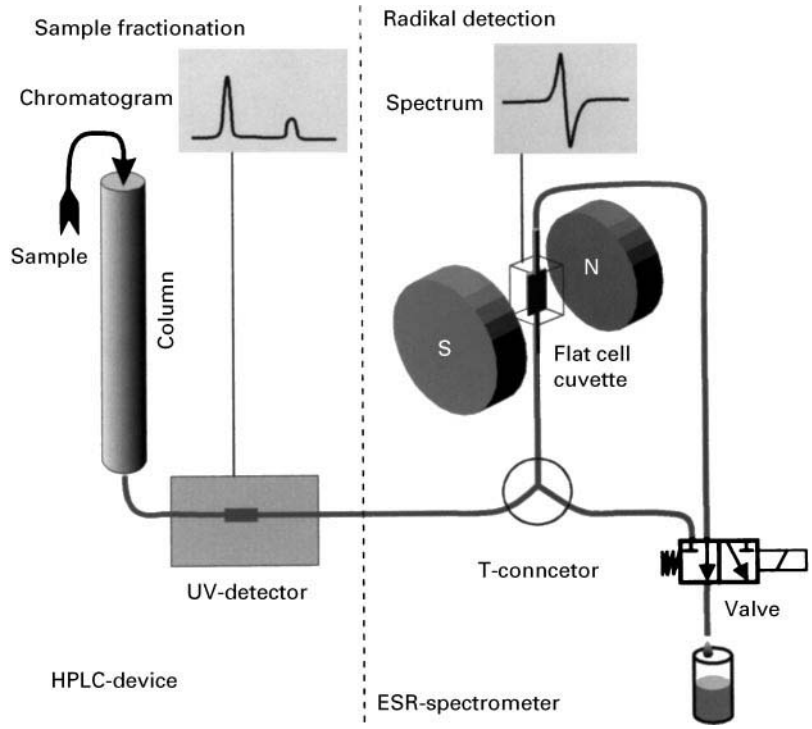

Figure 2 Instrumental set-up of synchronized online HPLC-X-band–ESR coupling (modified in accordance with Osterloh and Kroll (1998)).

'spin-trap'. To provide the possibility for monitoring such reactions online with an eluted fraction, certain supplementary equipment may be added to the whole instrumental set-up as shown in **Figure 3**.

Application Examples

The following practical examples should demonstrate the advantages in detection of free radicals in HPLC direct and online by ESR. One deals with separation of a synthesized product, while the other deals with a reactive, short-lived radical species.

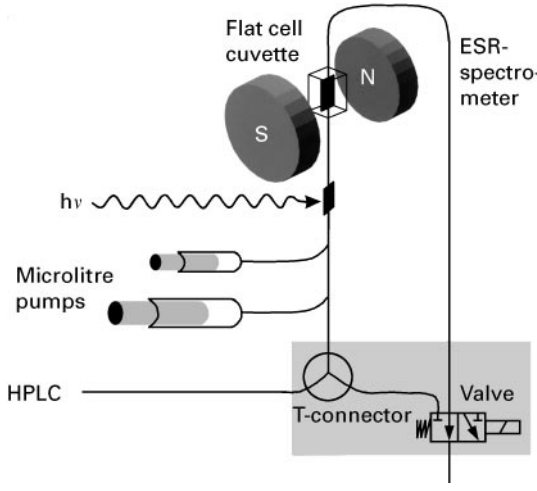

Figure 3 Attachment of supplementary equipment to the ESR spectrometer as radical detector.

Synthesis of Stable Radicals

Stable radicals such as nitroxyl compounds become increasingly of interest for process control of radical reactions, e.g. polymerization. This practical example demonstrates both the complexity of sample composition after such a synthesis and the changes owing to the instability of a single component. The starting point here is a cleavable biradical which decomposes immediately in aqueous solution.

Chemicals: 3-PCA-anhydride [**II**] was synthesized by the method of Gallez *et al*. using PCA [**I**]; 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl) and DCC (dicyclohexylcarbodiimide) (**Figure 4**).

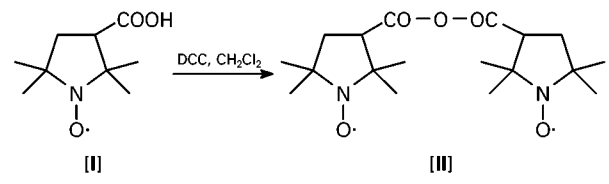

Figure 4 Synthesis scheme for PCA anhydride using DCC in purified methylenechloride.

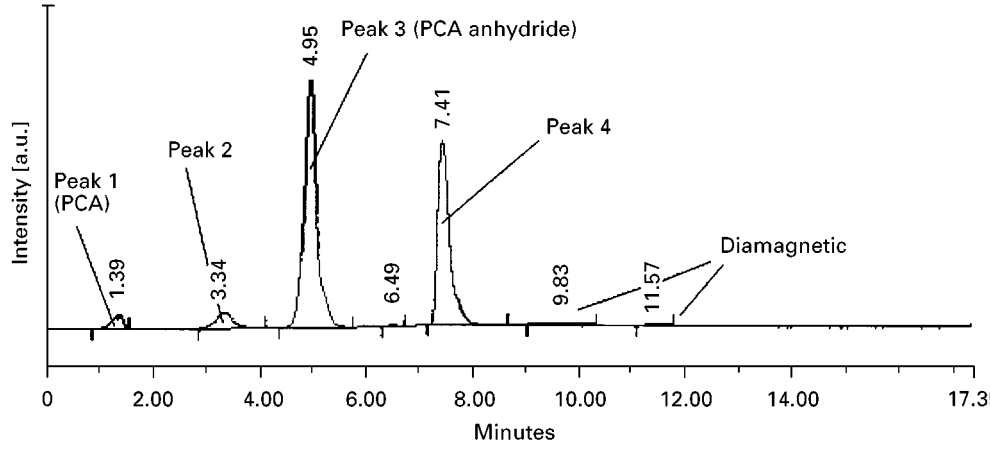

Figure 5 Chromatogram of synthesis product obtained by means of online coupled HPLC–ESR.

HPLC: Merck-Hitachi-HPLC with DAD (diode array detector), RP-18 (5 μm) column (125/4 mm) and methanol–water solutions (flow rate 1 mL min^{-1}, pH 7, isocratic as well as gradient mode).

ESR: Miniscope MS-100 desk X-band spectrometer (Magnettech GmbH, Germany). GC-MS, IR and NMR-techniques were also used for the identification of unknown products.

In following the synthesis, mixtures of PCA anhydride [**II**] were investigated by online coupled HPLC–ESR. This anhydride in practice represents a suitable tool for the spin labelling of macromolecules such as albumin. The purified synthesis product was analysed. In contrast to the available literature, the chromatogram obtained showed four ESR-active compounds after HPLC separation (**Figure 5**).

These were the PCA anhydride [**II**] (peak 3 with $t_R = 4.95$ min) and PCA [**I**] (peak 1 with $t_R = 1.39$ min) as expected as well as two new paramagnetic compounds with typical ^{14}N-hyperfine coupling (peak 2 with $t_R = 3.34$ min and peak 4 with $t_R = 7.41$ min). Further, ^{1}H-NMR, IR and GC–MS experiments showed the compound with $t_R = 7.71$ min (peak 4) to be PCA dicyclohexylureaamide. No other peaks gave an ESR signal.

Figure 6 represents the ESR spectra of the sample before and after chromatographic separations. Spectra of peak 1, 2 and 4 show a line triplet typical

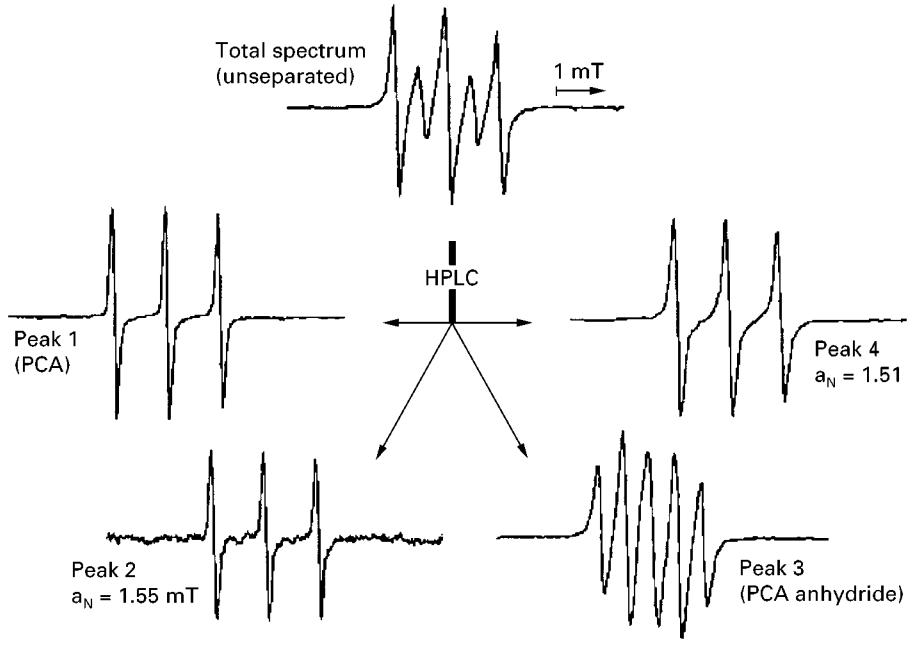

Figure 6 ESR spectra of the initial substance and four separated paramagnetic compounds.

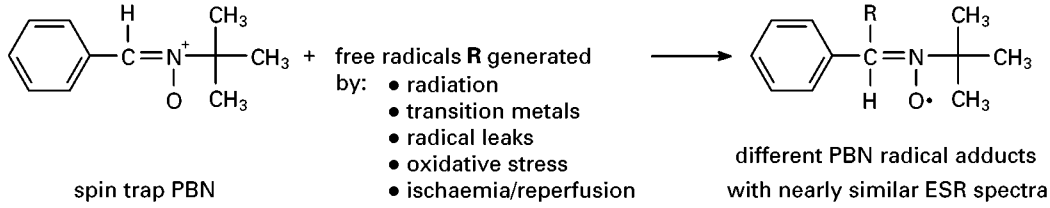

Figure 7 Scheme of formation of stable radicals in biological systems using PBN.

for nitroxyl radicals owing to the interaction between the unpaired electron and the nitrogen nucleus. The uneven number of protons and neutrons results in nuclear spin quantum numbers of -1, 0 and $+1$, in equal distribution. Those nuclei with a spin different from 0 cause a magnetic field recognized by the unpaired electron in addition to that generated by the magnet of the spectrometer. The result is a hyperfine splitting into three spectral lines as shown in the respective spectra. Peak 3 comprises the biradical PCA anhydride [**II**] with two nitroxyl groups in close vicinity. The ESR spectrum is determined by additional interactions between these two radical moieties. All the single spectra of the separated peaks add to the composite one seen with the initial unseparated sample.

Common Radical Analysis

A typical characteristic of most radicals is their high reactivity so they normally have a short life. As a consequence, they may be present only in rather low concentrations so they may be difficult to analyse. Therefore, the spin-trapping technique was developed to detect short-lived radicals. **Figure 7** shows an example of this technique in which the reagent PBN (α-phenyl-*N*-t-butylnitrone; not itself a radical) forms a stable adduct with a reactive, short-lived radical. The highly reactive compounds analysed here are inherently linked to the aging process in living tissues.

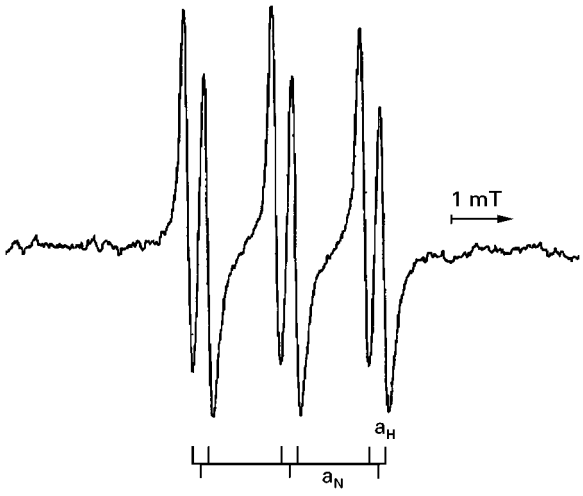

Figure 8 ESR spectra of a paramagnetic PBN adduct after HPLC separation measured by online HPLC–ESR coupling technique.

The spectrum in **Figure 8** shows, in addition to the hyperfine splitting owing to the nitrogen nucleus (a_N), an additional one caused by the hydrogen nucleus in the β-position to the unpaired electron (a_H). This spin trap does not necessarily allow differentiation between radical species so combination of a separation step with the spin trapping reaction could provide this kind of information.

Conclusions

The simple and straightforward coupling of HPLC and ESR causes problems owing to the necessity to synchronize the chromatographic and the spectroscopic techniques. The first example presented shows that it is impossible to distinguish between more than two similar paramagnetic compounds with either technique alone. Therefore, the direct combination of both techniques requires additional equipment to solve the problems encountered. The second example demonstrates a tool to identify low concentrations, but highly reactive radicals, in separate fractions.

Our modified online HPLC–ESR coupling technique represents a suitable tool to solve such problems without any significant time lag with a high signal sensitivity comparable to conventional cw-ESR. Furthermore, the technique presented prevents severe problems that frequently occur in stop-flow chromatographic applications.

See also: **II/Chromatography: Liquid:** Detectors: Fluorescence Detection; Detectors: Infrared; Detectors: Mass Spectrometry; Detectors: Refractive Index Detection; Detectors: Ultraviolet and Visible Detection; Nuclear Magnetic Resonance Detectors.

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Detectors: Evaporative Light Scattering

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The evaporative light-scattering detector evolved from the early work of Charlseworth and MacRae. The device consists of a spray system that continuously atomizes the column eluent into small droplets. The droplets evaporate, leaving the solute as fine particulate matter suspended in the atomizing gas. In practice, the column eluent passes into a concentric nebulizer where it is nebulized in a hot stream of gas that may be air or, if so desired, an inert gas such as helium or argon. The suspended particulate matter is then made to pass through an intense light beam from a source such as a helium–neon laser. The light scattered by the particles is viewed at 45° to the incident beam by means, for example, of a pair of properly placed optical fibres. The scattered light that enters the fibres is transmitted to a photomultiplier, the output of which is electronically processed and passed either to a computer acquisition system or to a potentiometric recorder. The evaporative light-scattering detector might be considered to be a form of transport detector where the transport medium is the nebulizing gas. A diagram of the light-scattering detector is shown in **Figure 1**.

The column eluent enters the centre orifice of a dual, concentric jet nebulizer where it meets a heated stream of nebulizer gas from the surrounding annular orifice. The gas flow rate is adjusted to provide a jet velocity that is just above the speed of sound. The stream of droplets that are produced normally have a relatively wide range of size distribution and pass down a heated tube, called the drift tube. In this tube the solvent evaporates, leaving the solute as residual solid particles still carried in the gas stream. It is clear that this type of detector cannot function effectively if solid involatile buffers are used in the mobile phase. After passing through the laser beam, the gas containing the solvent vapour and particles is aspirated through a simple water pump which safely disposes of both the solvent vapour and the solutes. The laser is employed as a convenient source of high intensity light and its coherence does not appear to confer any particular advantage on the detection system.

The amount of scattered light that is collected is related to the diameter of the particles, the wavelength of the incident light and the angle at which it is collected. It is not linearly related to the concentration of solute in the mobile phase, but varies as either the power or the exponent of the solute concentration. For a given set of operating conditions the droplet size will remain sensibly constant during the development of a chromatogram. Now the average diameter of the solid particles produced will be the average size of the droplets multiplied by the cube root of the solute concentration. Thus, if the solute has a concentration of 10^{-6} g mL^{-1}, the solute particles will be 100 times smaller than the size of the droplets and, assuming a common value for the mean diameter of the droplets of 20 μm, the solute particles will be 0.2 μm in diameter. Thus the mean particle diameter is of approximately the same order of magnitude as the wavelength of the scattered light.

Physical Properties of the Nebulizer

The intensity of the scattered light will be determined, among other factors, by the diameter of the solid